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L2          8622 S OSTEOCLASTS OR OSTEOCLAST
L3            84 S L1 AND L2
L4         93965 S PROGENITOR OR PRECURSOR
L5           520 S L4 AND L2
L6           497 S (PROGENITOR OR PRECURSOR)(P)(OSTEOCLASTS OR OSTEOCLAST)
L7            14 S L6 AND L1
L8         80361 S (JOINT OR KNEE(P)FLUID)
L9          3060 S (JOINT OR KNEE)(P)FLUID
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L13           13 S L4 AND L12

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L19         5788 S OSTEOCLASTS OR OSTEOCLAST
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Experimental Cell Research 77 (1973) 459–468

A FEULGEN-POSITIVE NUCLEOLUS

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SUMMARY

The Feulgen and Rossenbeck staining procedure reveals in all embryonic and adult cell types of the quail (*Coturnix coturnix japonica*) one or several chromatin condensations in the interphase nucleus. The Unna-Pappenheim technique, combined with RNAase treatment according to Brachet, shows that these chromatin masses are associated with the nucleolar RNA. Electron microscopic studies confirm this observation and the EDTA preferential staining procedure for RNP according to Bernhard makes it possible to distinguish three main types of nucleoli in the various tissues of the quail showing different patterns of RNA and DNA relationships. The functional significance of the large amount of nucleolus-associated chromatin in the quail, and of the more or less intimate relationships between RNA and DNA in the various types of nucleoli are discussed.

According to most investigators [14, 30], the nucleolus is Feulgen-negative. However, early studies on the nucleolus, involving Feulgen and methyl green staining procedures, revealed a dense perinucleolar shell of chromatin [8]. This nucleolus-associated chromatin was thereafter described in the electronmicroscope. Its contrast increases after ribonuclease digestion and disappears after DNAse treatment [3, 13]. This perinucleolar structure is considered as having a close relationship to the nucleolar function, and the nucleolus-associated chromatin is actually a part of the nucleolar apparatus [4, 7].

However, the presence of DNA within the nucleolus proper has been a controversial matter. Some DNA containing fibrillar material was found with the light microscope in the nucleolus [20, 23] but it was ultrastructural cytochemistry and electron autoradiography [13] which established clearly the presence within the nucleolus of chromatin strips; they are contiguous to the perinucleolar DNA, and appear as extensions of the nucleolus-associated chromatin [4].

The fact that the Feulgen reaction is negative in most nucleoli, though the presence of chromatin has been demonstrated with the electronmicroscope, suggests that the amount of nucleolar chromatin is below the limit of detectability by light microscopic procedures [7]. The RNP-containing structures are indeed the main component of the nucleolar apparatus, and the relationships between RNA and chromatin in vertebrate cell nucleolus are usually those represented in the scheme proposed by Bernhard & Granboulan [4].

The present paper deals with the description of a peculiar type of vertebrate cell nucleolus, which is to be observed in the Japanese quail (*Coturnix coturnix japonica*). In this species cytochemical procedures have shown that a large amount of chromatin is

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associated to the nucleolus in embryonic and adult cell types, making this organelle unusually large, and strongly Feulgen-positive [15]. Electronmicroscopic observations confirm that the chromatin is condensed in large clumps in the quail interphase nucleus, and the EDTA preferential staining procedure for RNA [2] shows different patterns of RNA and DNA relationships in the nucleoli according to the cell type. Various cell types from the chick have been treated by the same methods and compared with homologous quail cells.

MATERIAL AND METHODS

Embryonic and adult tissues of the Japanese quail (*Coturnix coturnix japonica*) and of the chick (*Gallus gallus*) have been treated for both light and electron microscopy.

Light microscopy

Fixations were carried out in Zenker's fluid and the slides stained according to the Feulgen & Rossenbeck [12] procedure for the specific detection of DNA. DNA and RNA were stained simultaneously by the Unna-Pappenheim [29] technique using pyronin and methyl green after fixation of tissues in Carnoy's fluid. Some preparations have been digested by RNase as controls for the localization of RNA in pyronin stained material [6].

Electron microscopy

Two different techniques have been used. The first is the classical technique: double glutaraldehyde-osmium tetroxyde fixation and uranyl acetate-lead citrate staining. The second is Bernhard's [2] regressive EDTA technique.

For classical observations, tissues were fixed in a 6% glutaraldehyde solution in 0.15 M Sorensen's phosphate buffer at pH 7.4 for 20 min, rinsed and postfixed for 1 h in 1% OsO^4 in the same buffer. The tissues dehydrated with alcohol were embedded in Epon. Ultrathin sections were cut with a Reichert ultramicrotome, and stained with uranyl acetate followed by lead citrate according to Reynolds [26].

For the RNP staining procedure, the tissues were fixed in 1.6% glutaraldehyde in phosphate buffer for 1 h without post-fixation in osmium tetroxyde. After alcohol dehydration the specimens were embedded in Epon according to the usual procedure. Ultrathin sections of silver-gold interference colour were stained with 5% uranyl acetate for 1 min, rinsed in distilled water, dried 10 min. Differentiation was carried out by floating the grids on 0.2 M EDTA, pH 7, between 5 and 30 min depending on the tissue. The grids were rinsed again and poststained with lead citrate for 5 min. Some grids are not treated by EDTA solution; they are stained with uranyl acetate for 1 min, followed by lead citrate for 5 min. This last procedure gave a good contrast of RNA and DNA containing structures.

RESULTS

Light microscopy

Comparative structure of quail and chick interphase nucleolus after Feulgen staining. The Feulgen reaction, when applied to both quail and chick embryonic or adult tissues, reveals important differences between the interphase nucleus in the two species. In quail cell nuclei, the chromatin is usually condensed in a large

Fig. 1. Sensory ganglion cells of a 7-day-old quail embryo. Feulgen staining. × 1 300.
Fig. 2. Sensory ganglion cells of a 7-day-old chick embryo. Feulgen staining. × 1 300.
Fig. 3. Embryonic mesencephalic cells of quail and chick at 7 days of incubation after the isotopic and isochronic graft of the right mesencephalic neural anlage of a 10-somite quail in a chick embryo host. Feulgen staining. × 1 300.
Fig. 4. Quail blastoderm (primitive streak stage). The nuclear Feulgen-positive condensations are large and irregularly shaped. Feulgen staining. × 1 300.
Fig. 5. Hepatocytes of a 15-day-old quail embryo. Several chromatin condensations are seen in the nuclei. Feulgen staining. × 1 300.
Fig. 6. Myocardic cells of a quail, 10 days after birth. Several chromatin condensations are seen in the nuclei. Feulgen staining. × 1 300.
Fig. 7. Hepatocytes of a 15-day-old chick embryo. Nucleolar associated chromatin is demonstrated (*arrows*). Feulgen staining. × 1 300.
Fig. 8. Adrenomedullary cell of a 12-day-old quail embryo. Glutaraldehyde-osmium fixation. Uranyl acetate-lead citrate staining. Compact nucleolus with a large amount of associated chromatin (*arrow*). × 11 600.
Fig. 9. Quail adrenomedullary cell after EDTA treatment. Nucleolus-associated chromatin is unstained while nucleolar RNP, located laterally is heavily contrasted. × 24 200.
Fig. 10. 13-day-old quail embryo metanephros. After EDTA treatment the centronuclear DNA condensation associated to the nucleolar RNA appears very faintly stained. × 19 800.
Fig. 11. Nucleolus of a 12-day-old quail embryo hepatocyte. A juxtaposition of RNA and DNA containing structures can be seen. Glutaraldehyde-osmium fixation. Uranyl acetate lead citrate staining. × 33 000.

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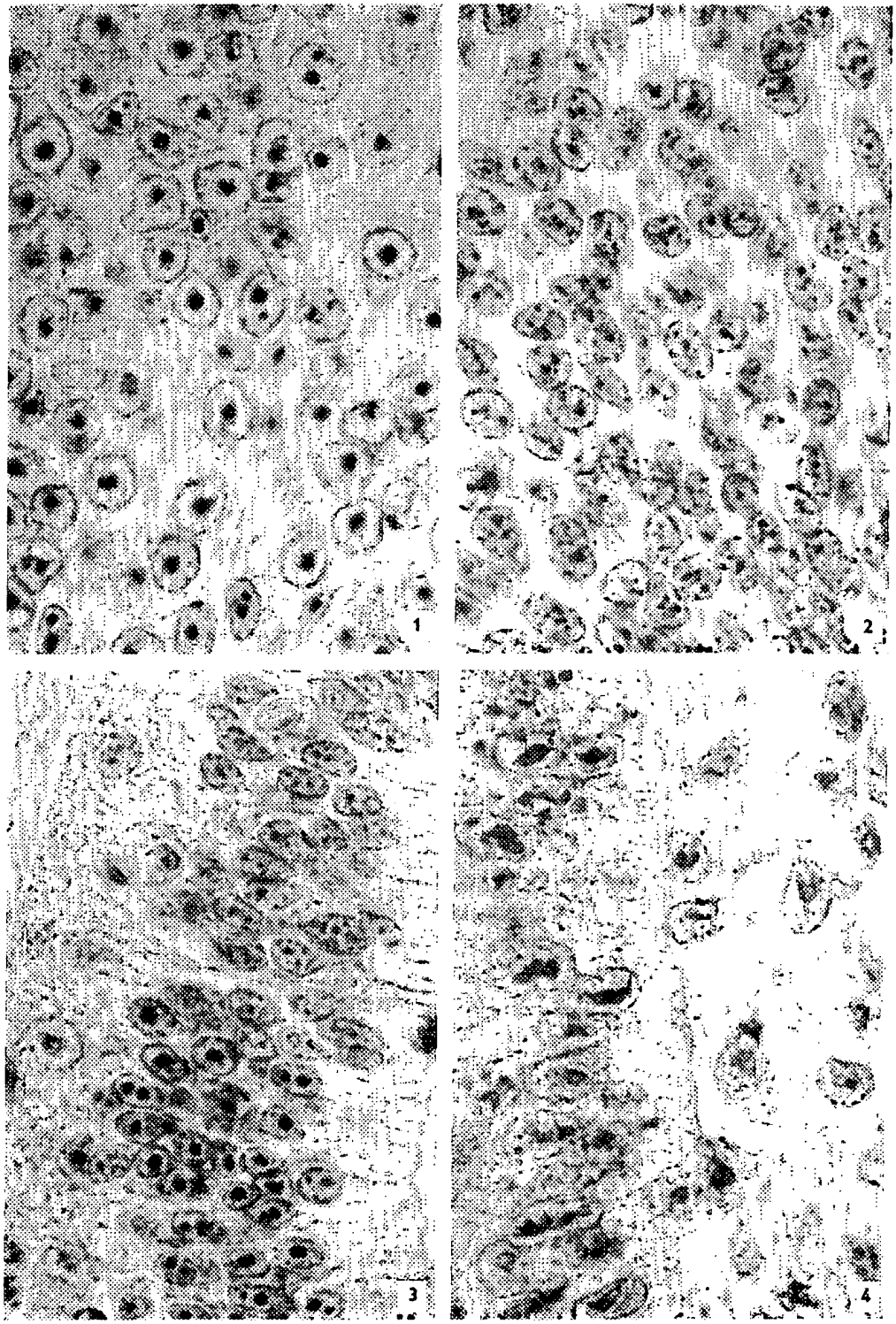

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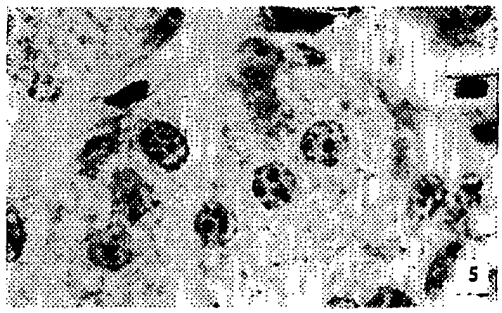

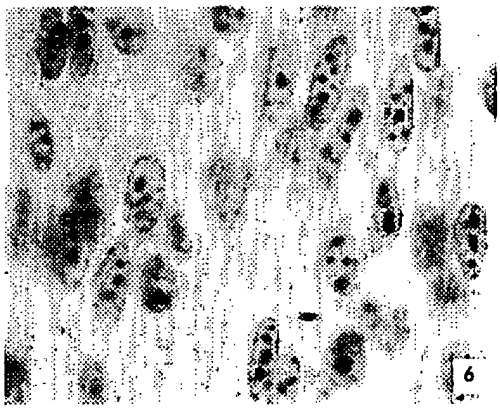

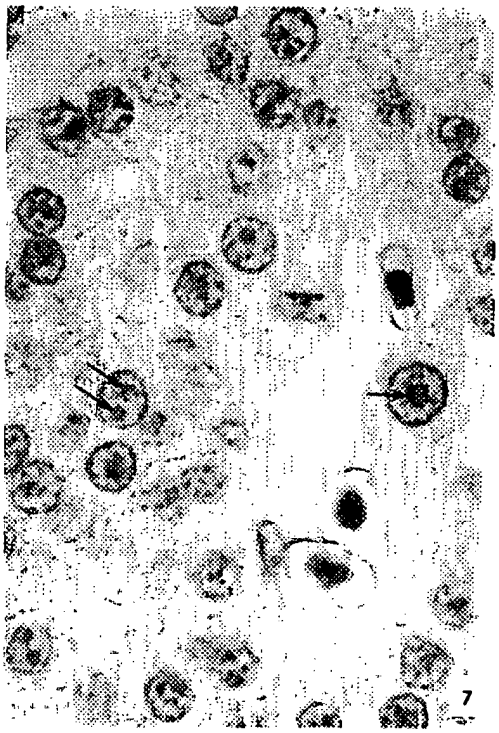

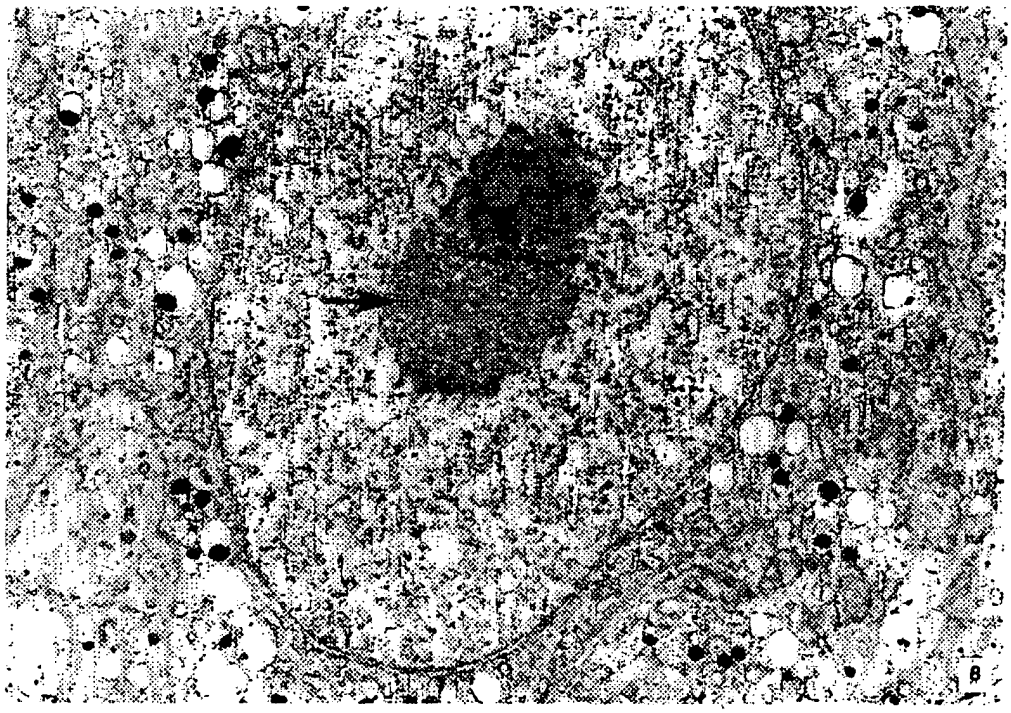

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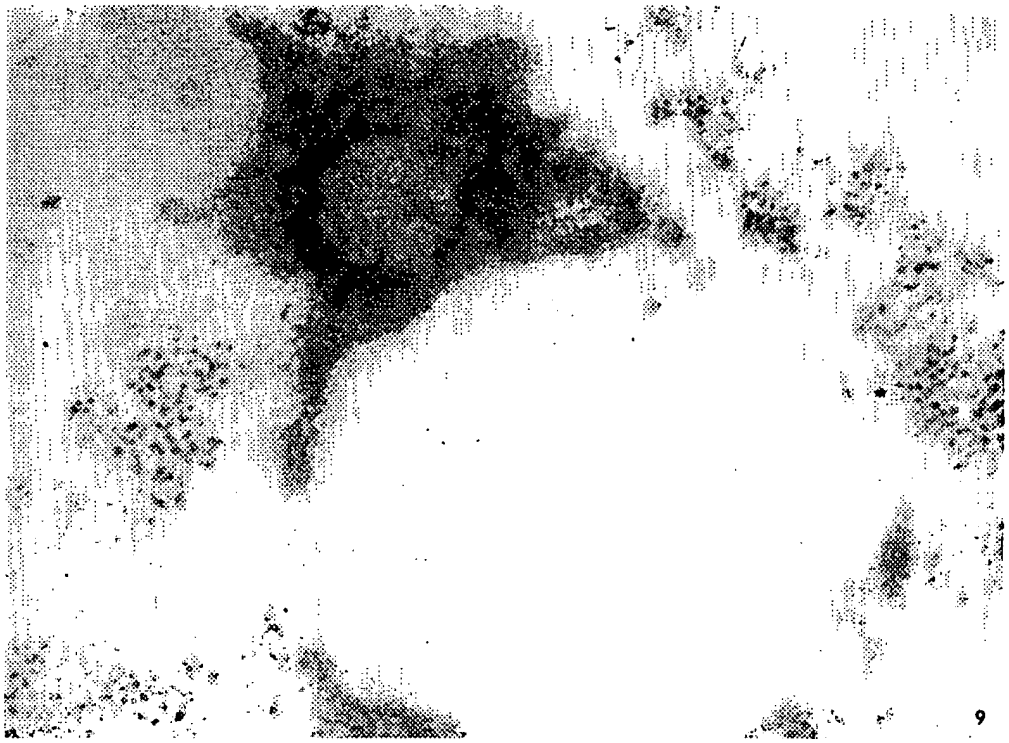

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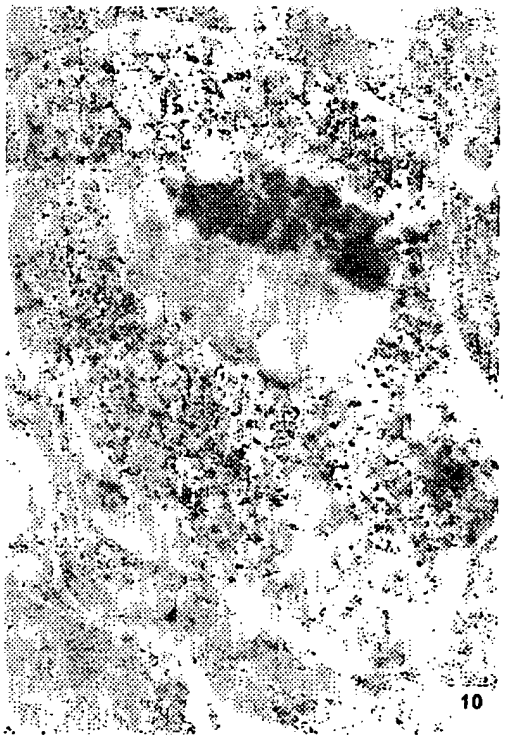

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central mass, strongly Feulgen-positive, around which the nucleoplasm is only slightly stained by Schiff reagent (fig. 1). In some cell types, the nucleus may contain several chromatin clumps, but in no cell type does the DNA appear evenly dispersed in the nucleoplasm. On the contrary, in the chick cell, the interphase chromatin forms a network homogeneously distributed in the nucleoplasm, with only small dispersed chromocentres (fig. 2). Thus, when stained by the Feulgen-reaction, quail and chick cells may easily be distinguished after experimental association in vivo (grafts) (fig. 3) or in vitro (organotypic culture) [15].

Variations in interphase nucleus structure of various quail and chick cell types. Some variations in nuclear features are observed in the various cell types of the quail. In most embryonic or differentiated cells, the nucleus contains one or sometimes two centronuclear chromatin clumps. Such is the case in blastomeres of early blastoderms and cells of the three germ layers in young embryos. Quail embryos from the primitive streak to the 5-somite stages have been observed in this respect. Their nuclei contain an especially large centronuclear chromatin mass with irregular outlines and a reticulated structure (fig. 4). In various differentiated tissues such as mesonerphos, metanephros, lungs, thyroid, parathyroid, suprarenal glands, neural anlage and differentiated nervous derivatives, etc., the chromatin condensations appear as compact, strongly Feulgen-stained structures with well-defined outlines (figs 1, 3). In the hepatocytes, there may be from 2 to 4 chromatin clumps some of them being attached to the nuclear membrane (fig. 5). The muscular cells have generally an elongated nucleus with 3, 4 and up to 5 heterochromatic condensations lined up along the nuclear axis (fig. 6).

In the chick, only small variations of the nuclear structure can be distinguished: in all cell types considered, the chromatin network is rather homogeneously distributed in the nucleoplasm (figs 2, 3). In some kinds of cells, for instance hepatocytes, where the nucleolar apparatus is especially developed, the shell of nucleolus-associated DNA is clearly shown up by the Feulgen reaction as a thin stained ring surrounding a clear region occupied by nucleolar RNA (fig. 7).

DNA and nucleolar RNA relationships as revealed by the Unna-Pappenheim staining procedure. The Unna-Pappenheim reaction has been applied to various embryonic and adult quail cells. The nuclear chromatin condensations, stained by methyl green, are closely associated with the pyroninophilic material, the latter disappearing when digested by RNase. In the hepatocytes, chromatin and RNA form large, irregularly shaped nucleoli in which green coloured and pyroninophilic material are distinct. In some other cell types, as mesonephritic tubules or young blastoderm cells for instance, the central nucleolar body is made up of closely intermingled structures stained green and red. Thus, it appears that a large amount of Feulgen-positive material forms a part of the nucleolus apparatus in all quail cell types. In chick cells, only a small amount of chromatin material is associated to the nucleolar RNA which forms a red staining spot the size of which varies according to the cell type. The associated chromatin is visible around the nucleolus as a thin ring of green staining material.

Electron microscopy

The association pattern of DNA and RNA containing structures in the nucleolus of the quail varies in different cell types as evidenced by the EDTA staining method. Chromatin appears bleached or very faintly stained

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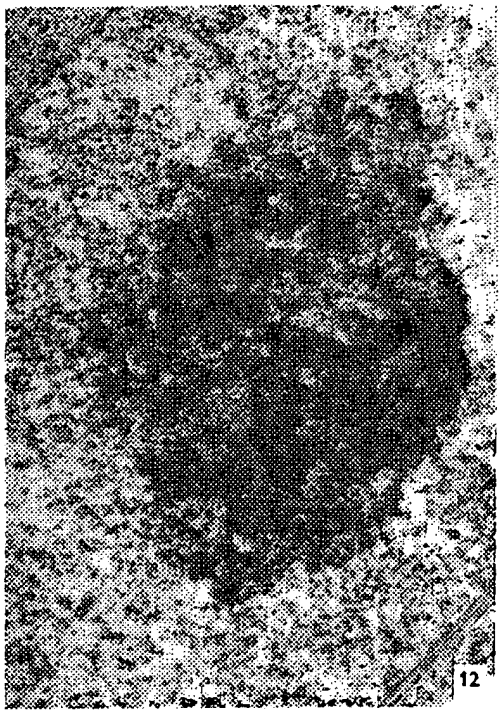

Fig. 12. Quail blastomere from primitive streak stage blastoderm. Glutaraldehyde-osmium fixation. Uranyl acetate-lead citrate staining. × 17 400.
Fig. 13. Quail blastomere from primitive streak stage blastoderm after EDTA treatment. RNA and DNA containing structures are closely intermingled inside the nucleolus which contains a large amount of RNP granular and fibrillar components. × 19 800.

whereas RNP show a dense contrast. Thus, the important contribution of DNA to the nucleolar apparatus appears very striking and, on the other hand, granular and fibrillar materials are more prominent than with the usual technique, since chromatin has lost its electron density.

Three main types of nucleoli have been distinguished in the quail.

Type 1: In numerous cell types, the nucleolus is a compact spherical or ovoid structure mainly composed of a DNA mass made up of densely coiled fibrillar material, with one, two, or three RNP-containing structures located laterally in relation to the DNA. The nucleolus is generally centronuclear and varies from 1.8 to 3 μm in diameter, with a most frequent size of 2.75 μm. This disposition can be observed for instance in adrenomedullary and adrenocortical cells, calcitonin cells of the ultimobranchial body, parathyroid and thyroid follicular cells, cells of distal mesonephric and metanephric tubules, connective cells of several organs (figs 8, 14*a*). In myocardic cells each of the DNA masses which were revealed by the Feulgen reaction, is seen to correspond to a nucleolus, with the general structure described as type 1, the respective amount of RNA and DNA being variable in the different nucleoli of the same cell.

The EDTA method shows in this kind of nucleolus a large zone occupied by unstained

chromatin, while RNP-containing structures are strongly contrasted (figs 9, 10). The nucleolar RNP fibrils and granules are arranged in various patterns; in many cases, the fibrillar material surrounds a less dense granular central zone (fig. 9).

Type 2: Another type of nucleolus is found in hepatocytes. Quail embryonic and adult hepatic cells show a large and usually irregularly shaped nucleolus in which RNP-containing structures are important and have variable connections with the DNA masses (figs 11, 14*b*). In many cases, nucleolar RNP form bridges between two chromatin condensations. The EDTA procedure reveals a large amount of nucleolar RNA with numerous strands of chromatin inside the RNP containing structures. In some cells, several nucleoli can be seen.

Type 3: The third nucleolar pattern observed in quail cells is very different from that previously described. The RNP-containing structures are located inside the centronuclear DNA mass as dispersed strands (figs 12, 14*c*). Due to their different electron density the simple glutaraldehyde fixation followed by the double uranyl acetate lead citrate staining makes it possible to distinguish clearly between these intermingled structures. This kind of nucleolus has been regularly encountered in the proximal thick wall tubules of mesonephros and metanephros, in epithelial cells of the embryonic duodenum and in the blastomeres of young blastoderms. The nucleoli of the blastomeres are very large and usually ovoid in shape. Their main diameter may reach up to 4 or 6 μm. The EDTA technique shows close relationships between RNP and chromatin (fig. 13). In blastomere nucleoli, the largest part of nucleolar material seems composed of RNP. The enlargement of the nucleolus in this cell type is due to the synthesis of a large amount of RNA. Fibrillar material and large fields of RNP granules, in which strands of bleached chromatin are included, are conspicuously visible. In blastomere nucleoli the RNP-containing structures form a continuous network in the meshes of which the associated chromatin is located. In mesonephric or duodenal cells the main component of the nucleolus is DNA inside which RNP strands appear discontinuous and scattered.

DISCUSSION

In the quail interphase nuclei, an important amount of chromatin is condensed in one or several heterochromatic masses associated with the nucleolar RNA. The relationships between nucleolar RNA and DNA vary according to the cell type and show three main patterns. The distribution of nucleolus-associated DNA in a thin perinucleolar shell as most usually observed in vertebrate cells [4] is never encountered in quail cells. The connections between DNA and RNA containing structures vary in the different nucleolus types: in type 1, the main central DNA mass has only a small surface of contact with the nucleolar RNA, and the closest relationships between RNA and DNA are established by the intranucleolar chromatin strands (fig. 14*a*). In the hepatocyte (nucleolus type 2), (fig. 14*b*) DNA and RNA are more closely intricated than in type 1. Nevertheless, the closest contact between chromatin and ribonucleoproteic components is realized in type 3 (fig. 14*c*). This pattern is found in cells with strong cytoplasmic basophilia, such as young blastomeres, and seems to coincide with an active ribosomal synthesis.

During synthesis of nucleolar RNA, it has been shown in mammalian cultivated cells that the transcription process occurs at the inner surface of the perinucleolar and along

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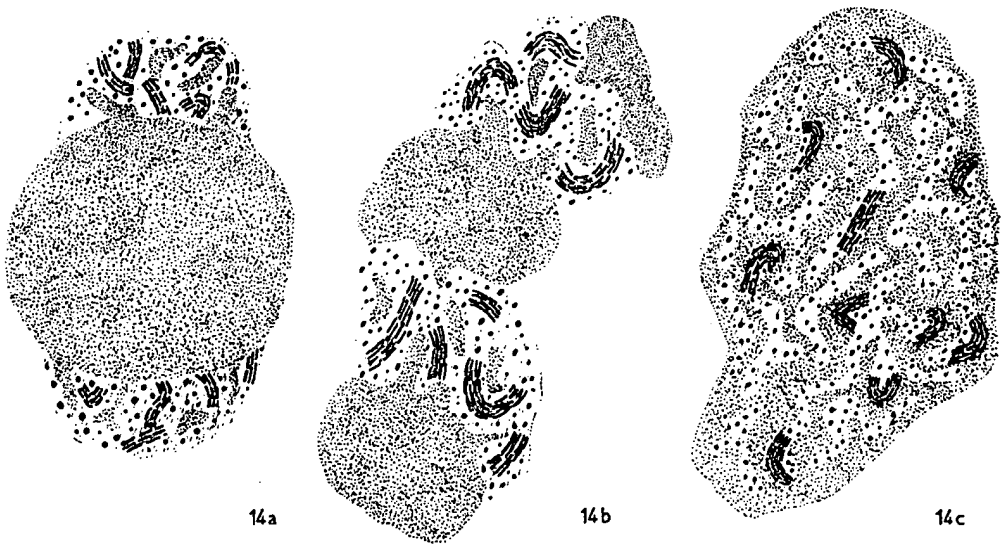

Fig. 14. Schematic drawings of ultrastructural organization of quail nucleoli. (*a*) Type 1 nucleolus of quail cells: Large chromatin condensation with lateral clumps of nucleolar material (granular and fibrillar containing structures and amorphous matrix) inside of which are located chromatin strands; (*b*) Type 2 nucleolus of quail cells: Observed in hepatocytes: several DNA condensations linked by nucleolar RNA; (*c*) Type 3 nucleolus of quail cells: RNA granules and fibrils are localized inside the large centronuclear DNA condensation.

the intranucleolar chromatin [13]. Thus the possible sites of RNA synthesis are conspicuously more abundant in type 3 nucleoli and especially in blastomere nucleoli than in various other cell types of the quail.

That all blastomeres of young blastoderms show the same nucleolar pattern indicates that during cell differentiation a change of nucleolar substructure occurs in most differentiating cell types. The inducing mechanism for such a change, the step of the differentiating process at which it occurs, and its functional significance in regard to the rate of protein synthesis would be interesting to know.

The functional significance of the high DNA content in the quail cell nucleolus is a rather questionable matter. The main function of the nucleolus being its involvement in ribosomal RNA synthesis, the question arises whether the considerable amount of nucleolus associated DNA in the quail results from an especially high amplification of ribosomal cistrons in this species. This appears most unlikely, since it has been demonstrated in numerous mammalian cells that the amount of rDNA which specifically codes for 18S and 28S RNA is only a very small part of the total DNA (about 0.005–0.01 %) [1, 21, 28]. In the chick [22] values ranging around 0.02 % of the total DNA have been reported to be hybridized with the RNA of ribosomes. In the quail as in other species, nucleolar DNA presumably consists not only of rDNA but also of other types of DNA.

The nucleolus-organizing chromosomes of the quail have been shown to be heterochromatic, late duplicating microchromosomes [10]. They differ from chick microchromosomes which, nucleolus-organizing, are [25] not heterochromatic [24] and not late replicating [5, 9, 11, 27]. The study of Comings & Mattoccia [10] reports observations on cultivated quail fibroblasts in prophase and pro-

metaphase. It seems likely that the nucleolus-organizing microchromosomes remain in a heterochromatic state during the interphase and form the large DNA clump associated with the nucleolar RNA observed in cells in vivo as well as in quail cells cultivated organotypically [15]. This disposition of chromatin material during the interphase is not found only in the quail. It has been observed in several species of birds which all belong to the Passeriform group [16]. However, not all passerines show a large amount of heterochromatin in their nuclei. The interphase chromatin shows the pattern observed in the chick, for instance in the cells of *Carduelis chloris*, *Carduelis carduelis* and others.

Besides the cytological interest presented by the Feulgen-positive nucleolus of quail cells, another aspect of this observation should be underlined. Due to their peculiar nuclear features, quail cells can be used as biological cell markers in embryological studies. The characteristics of quail nuclei persisting in organ culture conditions and in allografts of embryonic quail cells to the chick blastoderm, this labeling technique has, over previously used marking procedures, the advantage of being stable. The most commonly used labeling technique based on the incorporation of ^{3}H-thymidine are indeed only suitable for short time experiments, the nuclear marker becoming diluted through proliferation of embryonic cells. The use of quail cells as natural markers in heterospecific grafts makes it possible to recognize the origin of embryonic cells when they have reached a fully differentiated state. Thus, this labeling technique can be used in the study of various embryological problems, such as those related to cell migration, morphogenetic movements and intertissular or intercellular interactions during ontogeny [17, 18, 19].

The author wishes to acknowledge Professor W. Bernhard for his continued interest in this work and his critical reading of the manuscript.

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